

# Investigation into the Effect of Photodynamic Therapy and Cisplatin on the Cervical Cancer Cell Line (A2780)



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Published online December 30, 2020

## Abstract

**Introduction:** Cervical cancer is recognized as one of the major causes of mortality among elderly women. Although there are several different therapeutic worldwide guidelines, many researchers have focused on screening new methodologies and technologies to elevate the efficiency of cervical cancer treatment. The simultaneous use of photodynamic therapy (PDT) along with chemotherapy as cisplatin has achieved good aims in the treatment of cervical cancer.

**Methods:** A2780 cells were treated with cisplatin, photodynamic progress (laser with methylene blue as a photosensitizer compound) and a combination of cisplatin and PDT. The lithic effect of the laser, methylene blue and their combination and the IC50 value of cisplatin were calculated for each group. The amount of malondialdehyde (MDA) as membrane lipid peroxidation product and released lactate dehydrogenase was measured in the medium. The toxicity of each agent was evaluated by the MTT technique.

**Results:** The results show that a combination of PDT and chemotherapeutic agent cisplatin caused a twofold decrease in viable cervical cancer cells compared to each therapeutic progress. The combination of both laser therapy and cisplatin enhanced cancer cell membrane disruption by increased membrane lipid peroxidation and apoptotic enzyme activation by the elevation of lactate dehydrogenase activity.

**Conclusion:** The results indicated that cisplatin combined with PDT had a greater therapeutic effect on A2780 as a cervical cancer cell line. Therefore, PDT in combination with chemotherapy enhances the effectiveness of chemotherapeutic agents by the disruption of the cancer cell membrane and switching the apoptosis progress with less adverse effects.

**Keywords:** Cancer; Chemotherapy; Photodynamic therapy; Cisplatin; Cervical cancer; Laser.



## Introduction

Cancer is the first or second leading cause of death in people less than 70 years old according to the World Health Organization's (WHO's) report in 91 out of 172 countries studied in the year 2015.<sup>1</sup> According to the World Cancer Research Fund International, ovarian cancer ranks eighth as the most common cancer in women and 18th overall.<sup>1</sup> About 300 000 new cases of ovarian cancer were reported in the year 2018.<sup>1, 2</sup> Conforming to Globocan statistics, there were 382 000 new cases and 90 000 deaths from corpus uteri cancer in the year 2018.<sup>1</sup> Surgery, radiation therapy or/and chemotherapy, and their combination are some of the cancer treatments that are used. Because of the likelihood of metastasis, other methods are commonly used along with surgery. Although chemotherapy and radiography are very effective, damage to the surrounding

healthy tissue is one of their disadvantages. It is almost impossible to use the highest enough dose to kill all cancer cells without damaging normal tissue cells.<sup>3</sup> Chemotherapy is the use of anti-neoplastic agents to destroy cancer cells. This is aimed at disrupting cellular mechanisms and their reproduction.<sup>4</sup> Chemotherapy is a systemic treatment that has side effects such as nausea, vomiting, and hair loss.<sup>5</sup> Cisplatin has been used to treat various cancers.<sup>6</sup> This compound results in cancer cell death by interfering with DNA repair mechanisms and DNA damage.<sup>7</sup> Cisplatin forms cross-linkages within adjacent purines, mainly between 1, 2-d (GpG) and d (ApG) in DNA.<sup>8,9</sup> The function of this compound is also through the induction of oxidative and endoplasmic reticulum (ER) stresses.<sup>10,11</sup> Due to its positive charge, the interaction of cisplatin within the cell with protein

and RNA is also possible.<sup>12</sup> The cellular mechanisms suggested for cisplatin resistance include decreased drug accumulation through increased efflux and decreased uptake, detoxification by glutathione, increased DNA repairability, and the inhibition of apoptosis.<sup>13,14</sup> The side effects of cisplatin include dose-dependent nephrotoxicity,<sup>15</sup> hepatotoxicity,<sup>16</sup> cardiotoxicity,<sup>17</sup> anaphylactic reaction,<sup>18</sup> and hemorrhage.<sup>7</sup>

More than 3000 years ago, Egyptians, Indians, and the Chinese used the chemical reaction of some substances to light.<sup>19</sup> for the first time in 1900; Professor Hermann von Tappeiner from Germany witnessed the killing of living cells by a chemical reaction by a combination of light and a chemical substance. This was called Photodynamic Action. In the year 1970, a person named Dougherty used this phenomenon to treat cancer and called this new treatment photodynamic therapy (PDT).<sup>20,21</sup> Lipson et al reported in the 1960s<sup>22</sup> that the clinical use of PDT requires the presence of a specific photosensitizing, oxygen, and light agent that is consistent with the characteristics of photosensitizer absorption. When the photosensitizer is activated by the appropriate wavelength of light, it interacts with molecular oxygen to form a short-lived toxic species called single oxygen. This molecular oxygen is thought to mediate cell death.<sup>23</sup> On the other hand; treatment with chemicals can cause resistance as well as side effects. As known, other strategies, such as co-treatment, can achieve better response and thus less toxicity. This study attempted to evaluate the mechanism of the anticancer potential of PDT mediated by methylene blue as a photosensitizer alone or combined with a general cervical chemotherapeutic agent, cisplatin.

## Materials and Methods

### Cell Culture

In this study, a cervical cancer cell line (A2780) was used. The cells were cultured in an RPMI 1640 medium containing 10% fetal bovine serum at 37°C CO<sub>2</sub> 5%.

### Laser Treatment

The cells were first seeded in a 96-well plate (cells were cultured at a distance of one well to prevent radiation interference) and then they were exposed to a gallium aluminum arsenide diode Laser ( $\lambda=630$  nm, 5 mW; PI Dental Laser, Pioon, China) at 4 J/cm<sup>2</sup> (in a dark room).

### MTT Assay

To determine the toxicity of each agent alone, we calculate their toxicity by the MTT method alone. The basis of this method is based on the optical absorption of formazan sediments created by living cells. After the treatment of cells with different agents, the supernatant was removed and the culture medium containing 500  $\mu$ g MTT salt was added. This water-soluble salt was converted to a formazan insoluble precipitate by mitochondrial dehydrogenase enzymes. After 4 hours, the MTT salt medium was

removed from the cells and the DMSO compound was added to dissolve the formazan precipitate. Upon the dissolution of formazan sediments, a purple solution was formed which was absorbed in the 570 regions to measure its absorption and divided into the control group to be cell alive.<sup>24</sup> Cell viability results were shown as percentages in comparison with the control group. To quantify the sensitivity of selected cell types, the half maximal inhibitory concentration (IC<sub>50</sub>) which is the cisplatin concentration required for a 50% inhibition of cell growth was also calculated by GraphPad Prism 5.

### Methylene Blue Treatment

The methylene blue compound was used for sensitivity to light and cell viability was calculated relative to the compound at a concentration of 20  $\mu$ M after 2 hours. They were treated with methylene blue for 2 hours and then the medium containing serum was poured onto the cells and after 22 hours the percentage of living cells was calculated.

To evaluate the combined therapeutic effects, the cells were treated with different concentrations of cisplatin in the first step, and then they were treated with methylene blue for 2 hours. The culture medium containing methylene blue was discarded and the medium containing serum and the same concentration of cisplatin was added and mortality was measured by MTT after 22 hours.

### Measurement of Membrane Lipid Peroxidation

Malondialdehyde (MDA) concentrations were quantified using the lipid peroxidation assay kit (Kia-Zist, IRI). After each treatment, cell suspension was collected and centrifuged at 1230 rpm for 5 minutes to get the cell pellet, and the cell pellet was suspended in a cell lysis buffer. After centrifugation at 13 000 g for 10 minutes, a 200  $\mu$ L aliquot was assayed for MDA levels according to the lipid peroxidation assay kit protocol. The absorbance of the sample was read at 532 nm with a 96-well plate reader. The concentrations of MDA were determined from the standard curve.

### Measurement of Lactate Dehydrogenase Enzyme Leakage

Lactate dehydrogenase enzyme (LDH) enzyme leakage into the culture medium is a well-known indicator of cell membrane injury and cell cytotoxicity. It was measured using the LDH cytotoxicity assay kit (Pars-Azmoon, Iran). Briefly, 24 hours after the treatment, 100  $\mu$ L of the medium from each well was carefully transferred to the new 96-well plate, and 100  $\mu$ L of LDH reaction solution was prepared according to the manufacturer's instructions, added to each well. The 96-well plate was shaken at 37°C for 30 minutes, and then absorbance was measured at 490 nm with a 96-well plate reader. LDH release was expressed as a percentage of the control group.

### Statistical Analysis

The results were expressed as the mean  $\pm$  standard deviation (SD) value of 3 independent experiments. Data analysis was performed by using one-way ANOVA with Tukey post hoc test using GraphPad Prism<sup>®</sup> version 5.01 software (GraphPad Software, USA).  $P < 0.05$  was considered statistically significant.

### Results

In this study, A2780 cervical cancer cells were treated with cisplatin, photodynamic (laser with a light-sensitive methylene blue compound), and cisplatin with photodynamic, and mortality was calculated in these cells.

Figure 1 shows the effect of different laser and methylene blue treatments and the laser-methylene blue combination on A2780 cells. The methylene blue and laser groups alone did not differ from the control group, whereas the combination of laser and methylene blue groups showed a decrease in the viability of A2780 cells.

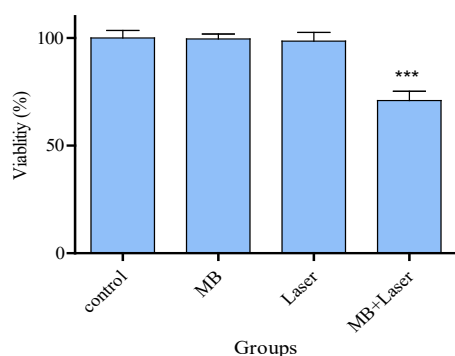
The viability of A2780 cells treated with different concentrations of cisplatin is plotted in Figure 2. The

concentration of 0.1  $\mu\text{g}/\text{mL}$  cisplatin was not significantly different from the control group, but with increasing cisplatin concentration to 1.6  $\mu\text{g}/\text{mL}$ , the percentage of cells decreased.

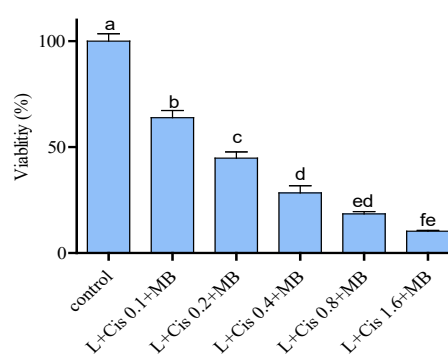
The results from IC50 for the combination of cisplatin alone and cisplatin with photodynamics are reported in Table 1. IC50 reported for cisplatin is approximately twice as high as IC50 for cisplatin, laser, and methylene blue.

Figure 3 shows the survival of A2780 cells in the cisplatin, laser and methylene blue, laser and methylene blue treatments in combination with cisplatin. At all concentrations of cisplatin examined in this study, the viability of A2780 cells treated with cisplatin decreased compared to the combination of methylene blue, laser and cisplatin concentrations. The cell viability of laser and methylene blue was lower than that of cisplatin at a concentration of 0.1  $\mu\text{g}/\text{mL}$  and slightly higher at the concentration of 0.2  $\mu\text{g}/\text{mL}$ .

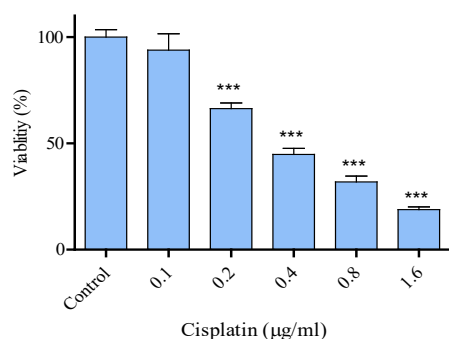
The MDA content as the end-product of lipid peroxidation in different groups is shown in Figure 4. Low-level laser, cisplatin and methylene blue



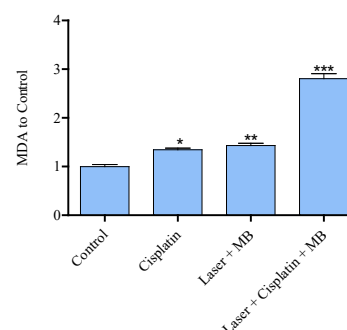
**Figure 1.** Graph of the Effect of Different Laser and Methylene Blue Treatments and the Laser-Methylene Blue Combination on A2780 Cell. Different groups are compared with the control group (\*\*\*) $P \leq 0.001$ .



**Figure 3.** Diagram of the viability of A2780 cells in cisplatin, Low-level laser and methylene blue, laser and methylene blue treatments with cisplatin. The presence of a common letter in both columns indicates no significant difference between the two groups ( $P \leq 0.05$ ).



**Figure 2.** The Viability of A2780 Cells Treated With Different Concentrations of Cisplatin. Different groups are compared with the control group (\*\*\*) $P \leq 0.001$ .



**Figure 4.** the Membrane Lipid Peroxidation Rate in Different Treatment Groups (cisplatin protection is the same as IC50 obtained from the previous step). All groups are compared with the control group (\*\*\*) $P \leq 0.001$ .

**Table 1.** IC50 Obtained for the Combination of Cisplatin Alone and Cisplatin With Photodynamic Therapy

	Cisplatin	Cisplatin + Laser	Cisplatin + Laser + Methylene Blue
IC50 (µg/mL)	0.3553	0.3043	0.1680

groups simultaneously increase the rate of membrane peroxidation by approximately 30%. This rate in cisplatin treatment alone was not greater than 20% like the laser with methylene blue group.

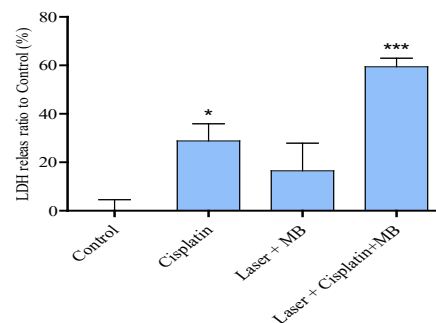
The release of LDH in different treatment groups is stated in Figure 5. LDH in the control group was zero. The release rate of LDH in cisplatin alone was greater than 20% and in the laser with methylene blue less than 20%.

## Discussion

PDT is one of the novel therapeutic concepts in cancer therapy methods. New investigations have focused on the mechanisms of biological activity of PDT, especially in cancer therapy. Chemotherapy based on drugs with a metal ion core, such as Cisplatin, is generally administered by blood infusion.<sup>25</sup> It diffuses through the cell wall into the cell and due to the decrease in intracellular chloride concentration, the cisplatin hydrolysis reaction occurs.<sup>26</sup> Cisplatin is bound to phospholipid and phosphatidylserine in the cell membrane. Besides, the binding of this compound is carried out in cellular cytoplasmic RNA and sulfur-containing molecules. The process of inducing apoptosis and cell necrosis is due to cisplatin binding to DNA and impaired proliferation, transcription and repair.<sup>27</sup> Research on the mechanism of the action of laser light has been ongoing for about fifty years. Various ways have been suggested that lasers directly or indirectly generate hydrogen peroxide ( $H_2O_2$ ) in the cell. Therefore, lasers affect biological activity by producing a small amount of  $H_2O_2$ .<sup>28</sup> On the other hand, many studies have shown the amount of  $H_2O_2$  in cancer cells has increased rather normal cells, which may play a role in DNA alterations,<sup>29</sup> apoptosis resistance,<sup>30</sup> cell proliferation,<sup>31</sup> angiogenesis<sup>32</sup> and metastasis.<sup>33</sup> Furthermore, high levels of  $H_2O_2$  can predispose the cell to death. Somehow, it can be stated that  $H_2O_2$  has a dual role in cancer.<sup>34</sup> PDT is a phototherapy method in which visible light and medication are used simultaneously. Activation of the light-sensitive drug by visible light results in the production of cytotoxic oxygen species and free radicals. These free radicals cause cell death directly via necrosis or apoptosis, vascular obstruction, and destruction of tumor tissue by inflammatory and immunologic factors. Generally, PDT operates through the production of reactive oxygen after the drug is delivered to the cancer patient and light irradiation.<sup>35</sup>

Among various chemotherapeutic agent treatments in patients with cervical cancer, cisplatin, according to Alberts et al, is the only toxic active agent against this

cancer. However, they state that the short life span and the development of tumor resistance to cisplatin are the disadvantages of this drug combination.<sup>36</sup> As the results of this study show, the viability of the A2780 cells as the in vitro model of cervical cancer decreased in elevating the concentrations of cisplatin. Buxton et al investigated the combination of bleomycin, ifosfamide, and cisplatin in patients with cervical cancer, and Cisplatin was active against this type of cancer. The side effects of this treatment were also reported.<sup>37</sup> In 2006, Crescenzi et al conducted a study on the effect of cisplatin and gemcitabine in the presence of PDT on H1299 cells. The results showed the synergism effect of cisplatin and PDT.<sup>38</sup> Rose et al compared radiotherapy and cisplatin in women with cervical cancer and reported favorable anticancer outcomes.<sup>39</sup> Zhou et al examined the effect of timeless on cell proliferation and cisplatin sensitivity in cervical cancer and reported that timeless regulated cisplatin cell proliferation and susceptibility, and could serve as an attractive target for cis-sensitizer. Platinum was involved in cervical cancer.<sup>40</sup> In a similar study, the destruction of cervical cancer cells was investigated using PDT in 2004. The result of this study showed that Photogem (photosensitizing agent whit hematoporphyrin derivative) is specifically dependent on the cancer tumor and that necrosis is due to plasma membrane damage. The researchers asserted that the main mechanism of the anti-tumor effect of PDT using Photogem was likely.<sup>41</sup> In 2014, de Freitas et al conducted a study into the combined therapeutic effects of PDT and cisplatin on the HaCaT, siHa, and C-33A cell lines. The results showed that the effect of PDT with cisplatin was greater than the treatment with each of the agents individually. The order of treatment with cisplatin and PDT was also important, and the cells that were first irradiated and then treated with the drug showed higher mortality.<sup>42</sup> In 2004, Crescenzi et al conducted a study on MCF-7 cells and examined the combined therapeutic effects of cisplatin and PDT. In the study, a diode laser with a



**Figure 5.** The Lactate Dehydrogenase Release Rate in Different Treatment Groups (cisplatin protection is the same as IC50 obtained from the previous step). All groups are compared with the control group (\*\*\*) $P \leq 0.001$ .

wavelength of 805 nm was used and the results showed the effects of cisplatin synergism and PDT.<sup>43</sup> Similarly, a study on the simultaneous effects of cisplatin and PDT on the in-vivo and in-vivo environment showed that a combination of both treatments was more effective than the two treatments individually.<sup>44</sup> In this study, as can be seen, concurrent combination therapy was better than individual therapy for each of the agents. The IC50 level in the combination of cisplatin, laser and MB agents was about 2 times lower than that of cisplatin alone, which indicates the superiority of the use of cisplatin, laser and MB complex over cisplatin alone in observing the desired therapeutic effect. In more previous research studies, a phenomenon known as the Warburg effect suggested that the rate of metabolism of glucose-to-lactate conversion (aerobic glycolysis) occurs about 10-fold higher in tumor tissue than in healthy tissue.<sup>45,46</sup> Lactate dehydrogenase is an enzyme in the glycolytic pathway that is expressed in many tumors, and its activity increases.<sup>47-50</sup> The results of the current study demonstrated that in a combination of laser, cisplatin, and MB, the rate of release of LDH was greater than that of cisplatin alone or MB with the laser. The effect of cisplatin, laser, and MB synergism in the treatment of cervical cancer cells is thought to be due to the fact that PDT produces reactive oxygen species<sup>35</sup> such as superoxide, hydrogen peroxide, and hydroxyl radicals.<sup>51</sup> These compounds, in addition to damage to DNA, protein and lipid,<sup>52,53</sup> cause cell membrane disruption.<sup>41</sup> The results of the present study suggest that cisplatin combined with PDT has more desirable effects than the treatment of each of the agents alone for cervical cancer therapy through enhancing the cancer cell membrane lipid peroxidation and the activation of apoptotic enzymes.

### Ethical Considerations

This study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.REC.1398.140).

### Conflict of Interests

The authors declare no conflict of interest.

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